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In-vitro and in-vivo antagonistic action of an anti-amylin Spiegelmer

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In vitro and in vivo antagonistic action of an anti-amylin Spiegelmer

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Short title: Spiegelmer antagonizes central amylin actions

Contents

Abstract

The anorectic and dipsogenic effects of the pancreatic hormone amylin are mediated by the area postrema and the subfornical organ. We tested the effectiveness of a new amylin antagonist, a so-called RNA Spiegelmer, by electrophysiological *in vitro* recordings from the rat subfornical organ and by immunohistological c-Fos studies in the area postrema. Amylin`s excitatory effect on subfornical organ neurons was blocked by the anti-amylin Spiegelmer. Peripheral administration 5 h prior to amylin also suppressed the amylin-induced activation (c-Fos expression) in the area postrema. The biostable anti-amylin Spiegelmer may be therapeutically beneficial in conditions associated with high plasma amylin levels, such as cancer anorexia occurring during certain pancreatic tumors.

Key words: *amylin, Spiegelmer, NOX-A42, area postrema, subfornical organ, electrophysiology, immunohistochemistry*

Introduction

Amylin is a 37-amino acid peptide, co-secreted with insulin from pancreatic ß-cells in response to food intake [1]. Exogenous amylin potently reduces food intake [2, 3], gastric emptying and glucagon secretion [4, 5]. Peripheral amylin's anorectic action seems to be mediated by the area postrema [6, 7], a circumventricular organ lacking a functional blood-brain-barrier [8]. In addition to its anorectic effect, amylin may also contribute to prandial drinking most likely via an action on angiotensin II sensitive neurons of the subfornical organ [9]. The subfornical organ also belongs to the circumventricular organs and, similar to the area postrema, expresses a high density of amylin binding sites [10, 11, 12]. As a member of the calcitonin family, amylin binds to calcitonin receptors that exhibit an affinity for amylin when co-expressed with receptor activity modifying protein (RAMP) 1 or 3 [13, 14].

In previous electrophysiological and immunohistological studies we characterized the effects of amylin on neuronal activity in the area postrema [15, 16] and the subfornical organ [9, 17]. In both structures, amylin induces dose-dependent excitatory effects that are blocked by the peptidergic amylin antagonist AC187. AC187, a truncated analogue of salmon calcitonin [18], reduces the excitatory effect of amylin on area postrema and subfornical organ neurons [9, 16], the amylin-induced c-Fos expression in the area postrema [15] and attenuates peripheral amylin's anorectic effect when infused directly into the area postrema [7]. Recently, a new class of hormone antagonists has been developed, the so-called Spiegelmers (German: Spiegel = mirror). Spiegelmers are mirror image L-oligonucleotides with high affinity towards a given target molecule, leading to a blockade of its biological activity [see ref. [19] for review]. Due to their L-ribose containing sugar-phosphate backbone, Spiegelmers are highly resistant to nuclease degradation and remain stable for more than sixty hours in biological fluids such as human plasma [19]. In addition to their usefulness as experimental tools, they may also be used as therapeutic agents. It has already been demonstrated that the anti-ghrelin SPM NOX-B11 when infused into the tail vein, suppresses ghrelin-induced food intake and c-Fos

induction in the arcuate nucleus in rats [20]. Together with the observation that chronic NOX-B11 administration lowers body weight [20, 21], it may be beneficial for the treatment of obesity.

The aim of the present study was to provide functional *in vitro* and *in vivo* evidence that a newly developed anti-amylin Spiegelmer antagonizes the effects of amylin on area postrema and subfornical organ neurons. We used two different approaches. (1) In electrophysiological single cell recordings obtained from slice preparations, we investigated the ability of the anti-amylin Spiegelmer to block the excitatory action of amylin on subfornical organ neurons *in vitro*. (2) In immunohistological studies we determined whether the anti-amylin Spiegelmer blocks the amylin-induced c-Fos expression in the area postrema under *in vivo* conditions.

Methods

The anti-amylin Spiegelmer used in this study is a 42 nucleotide L-RNA with the sequence 5'-GGACUGAUGGCGCGGUCCUAUUACGCCGAUAGGGUGAGGGGA-3'. The molecule is a variant of the previously published Spiegelmer STAR-F12Δ43-48, whereby the adenosines at positions 30 and 35 were substituted to uridines [22]. The biologically inactive control Spiegelmer has the arbitrary sequence 5'-UAAGGAAACUCGGUCUGAUGCGGUAGCGCUGUGCAGAGCU-3'. The Spiegelmer used in the *in vivo* studies (NOX-A42) was modified with a 40 kD polyethylene glycol (PEG) moiety to reduce renal excretion [21]. All Spiegelmers were synthesized at NOXXON Pharma AG.

Male adult Wistar rats (200-300 g) were used for all experiments. Rats had *ad libitum* access to standard laboratory rat chow (890 25 W16, Provimi Kliba, Gossau, Switzerland) and water, unless stated otherwise. The animals were maintained in a temperature-controlled room on an

artificial 12:12-h dark-light cycle (21 ± 1°C, lights on at 3:00 AM) and adapted to the housing conditions and handling for at least 2 weeks before the start of the experiments.

The slice preparation of the subfornical and the electrophysiological recording technique were identical to the procedures described previously [9, 17]. Briefly, extracellular recordings of action potentials from spontaneously active subfornical organ neurons were obtained using glass coated platinum-iridium electrodes. After recording the baseline activity of a single neuron for at least 5 min, stimulations were conducted by switching to a superfusion solution (10 ml) containing the investigated drugs. The following stimuli were applied: 1. rat amylin (10^{-7} M, Bachem AG, Bubendorf, Switzerland), 2. rat amylin (10^{-7} M) plus anti-amylin Spiegelmer (50 x 10^{-7} M) and 3. rat amylin plus inactive Spiegelmer (50 x 10^{-7}M). The Spiegelmers were dissolved in distilled water and were pre-incubated with amylin for 15 min at room temperature before the start of the superfusion. When amylin was applied alone the amylin solution was kept under the same conditions. To exclude that the Spiegelmers cause any changes in the discharge rate, control superfusions of these substances (50 x 10^{-7} M) were applied without amylin. Action potential recordings were analysed using the software Spike2 (Cambridge Electronic Design, England). From the continuously recorded rate meter counts, the average discharge rate of each neuron was evaluated for 60 seconds prior to the stimulus. This value (spontaneous discharge rate) was used to normalize changes in firing rate, expressed as % change of the spontaneous discharge rate. If the averaged change of discharge rate during the amylin response was larger than ± 20% and ± 0.5 spikes/s, the neuron was considered amylin sensitive. In addition to the mean change of the discharge rate during the entire responses, the peak values of the responses were calculated on a basis of a 30 s interval during which the firing rate was maximal. Finally, the duration between the application of the drugs and the onset of effect (latency) and the duration of the entire responses were determined. The parameters of the electrophysiological responses were expressed as means ± standard errors (M ± SEM).

For the immunohistological c-Fos studies four groups rats (200-230 g) were used (n=5 per group). All animals were food deprived for 26 h beginning at dark onset. After 19 h of fasting, i.e. 5h before subsequent dark onset, two groups were pre-treated with NOX-A42 (3mg/kg i.v. into the tail vein), while the two other groups received control infusions of saline. Ten minutes before dark onset, one Spiegelmer and one saline treated group were injected with amylin (20 μg/kg s.c.), while the two remaining groups received saline. Two hours after these treatments, rats were deeply anaesthetized (pentobarbital 100 mg/kg, i.p.) and transcardially perfused with ice-cold sodium phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS. The immunohistological c-Fos staining was identical to the method described previously [23].

In the electrophysiological studies statistical differences between the responses were evaluated by Friedman Repeated Measures ANOVA on Ranks followed by Student-Newman-Keuls method for multiple pairwise comparison. For quantification of the c-Fos responses, c-Fos immunoreactive (c-Fos-IR) cells were counted for each animal from xx slices of the area postrema (according to the brain map of Paxinos and Watson [24]). All slices from each animal were taken from corresponding rostrocaudal levels in order to exclude quantitative differences due to variations in the cross sectional area. The data are expressed as average number of cells/section ± SEM. Differences between the groups were evaluated by one way ANOVA and the least significant difference pairwise multiple comparison test. For all statistical tests $P < 0.05$ was considered significant.

Results

Only amylin sensitive subfornical organ neurons were included in the electrophysiological analysis to test for the blocking capacity of the anti-amylin SPM. In total, six amylin responsive cells have been successfully tested with all three stimuli (amylin, amylin/anti-amylin Spiegelmer and amylin/inactive Spiegelmer). In contrast to the inactive Spiegelmer, the anti-amylin Spiegelmer effectively blocked the amylin-mediated excitations in the subfornical organ. Figure 1a illustrates a typical recording of an amylin-sensitive subfornical organ neuron demonstrating the blockade of amylin's excitatory action when co-applied with anti-amylin Spiegelmer, while the inactive Spiegelmer did not affect the amylin-induced response. The average excitatory effect of amylin calculated from the mean increases in firing rates of all tested neurons was significantly higher than the averaged response observed after a co-application of amylin and the active Spiegelmer. The mean excitatory response after application of amylin plus inactive Spiegelmer was similar to that of amylin alone. Table 1 summarizes all mean effect parameters for the different stimuli. Neither the active SPM (n=3) nor the inactive SPM (n= 3) affected neuronal activity when superfused alone (Fig. 1b).
In experimentally independent *in vivo* studies we confirmed the amylin antagonistic action of the Spiegelmer NOX-A42, which significantly attenuated the amylin-induced activation of AP neurons as detected by c-Fos expression. Almost no activated neurons were observed in saline/saline treated animals and in NOX-A42/saline treated rats (Fig. 2). While saline/amylin treatment lead to abundant c-Fos positive nuclei, significantly fewer cells were detected in NOX-A42/amylin injected animals. In the Spiegelmer pretreated group the amylin-induced c-Fos response in the AP was decreased by 54 % relative to the saline/amylin injected animals (Fig. 3).

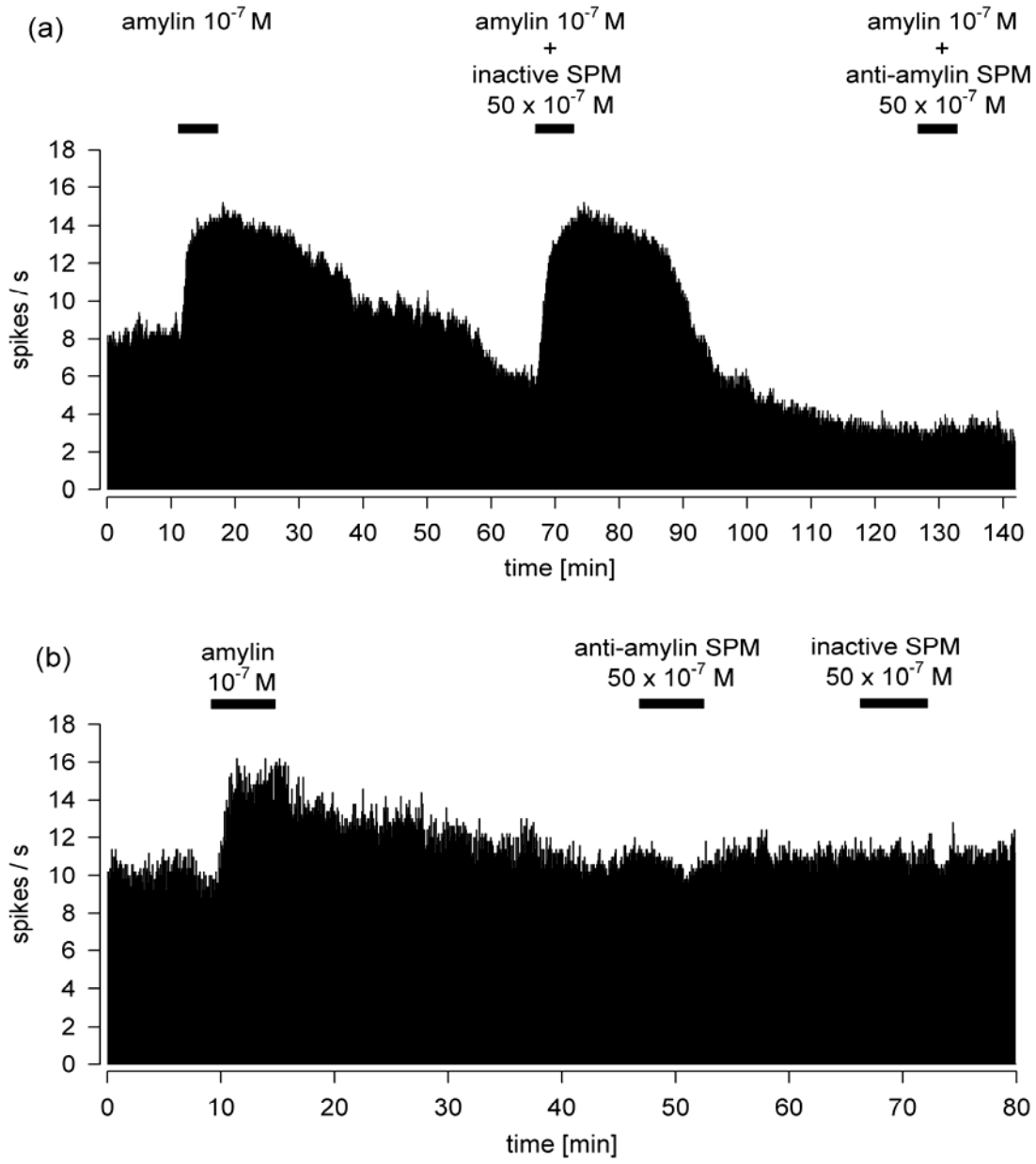

Fig. 1. a) Continous rate meter recording of a spontaneously active neuron from the rat subfronical organ. The amylin-induced excitation was antagonized by co-application of the anti-amylin Spiegelmer, while the biologically inactive Spiegelmer did not affect the amylin-mediated excitation. **b)** Continous rate meter recording of a spontaneously active neuron from the rat subfronical organ. Neither the inactive SPM nor the anti-amylin Spiegelmer affected neuronal activity when superfused alone.

Table 1. Effect parameters of the electrophysiological responses of rat subfornical neurons

Effect parameter	amylin	amylin + inactive Spiegelmer	amylin + anti-amylin Spiegelmer
Mean latency [s]	74.5 ± 23.6 [a]	84.3 ± 28.9 [a]	36.3 ± 11.3 [a]
Mean response [%]	45.4 ± 5.8 [a]	44.8 ± 11.9 [a]	4.8 ± 3.9 [b]
Mean response [spikes/s]	3.4 ± 0.4 [a]	3.4 ± 0.6 [a]	0.4 ± 0.2 [b]
Peak response [spikes/s]	4.7 ± 0.4 [a]	4.7 ± 1.0 [a]	0.8 ± 0.3 [b]
Mean response duration [s]	1142.5 ± 211.5 [a]	1171.2 ± 220.5 [a]	381.2 ± 37.6 [b]

Parameters are given for the responses induced by amylin (10^{-7} M) when superfused alone and in combination with inactive Spiegelmer or the anti-amylin Spiegelmer (both 50 x 10^{-7}M). Anti-amylin Spiegelmer significantly blocked the amylin-induced excitatory response compared to superfusion of amylin alone or in combination with the inactive Spiegelmer. Different letters indicate significant differences ($p<0.05$). Values are means ± SEM (n=6).

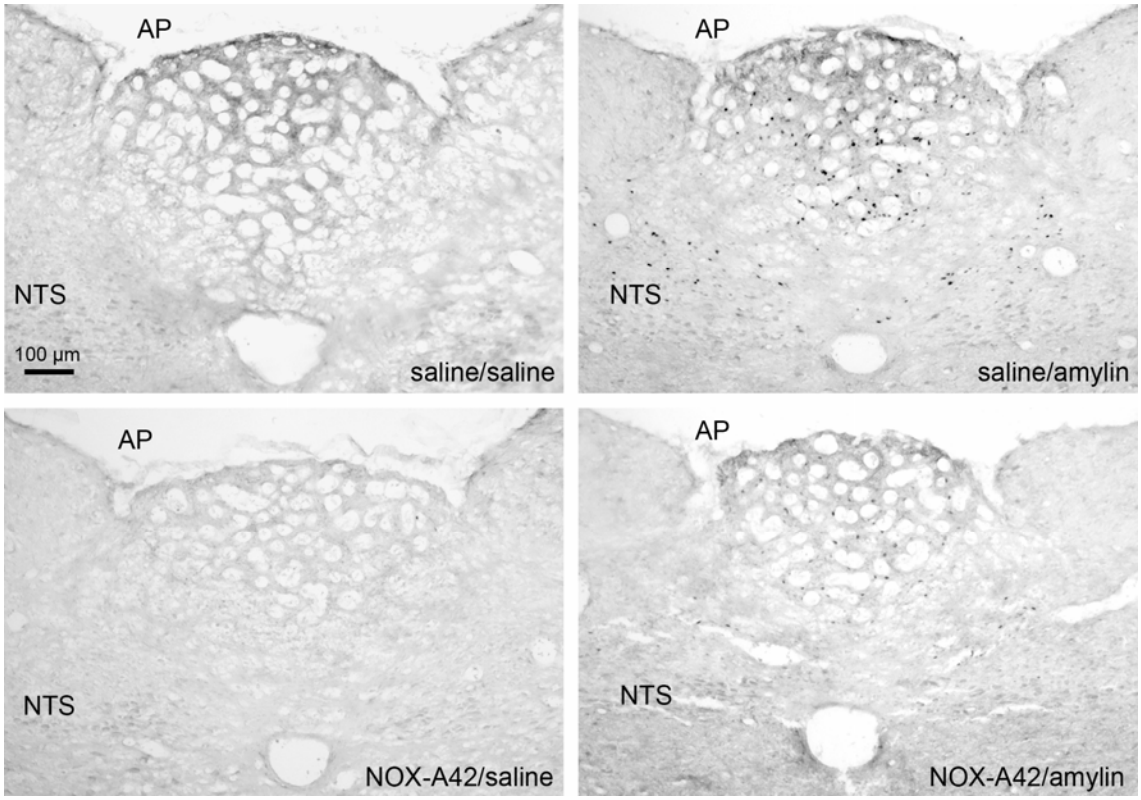

Fig. 2. Immunohistochemical stainings of 20 µm thick coronal sections of the area postrema processed for c-Fos after injections of saline/saline (control), NOX-A42/saline, saline/amylin and NOX-A42/amylin treated animals. NOX-A42 (3 mg/kg i.v.) reduced the amylin (20 µg/kg s.c.) induced c-Fos expression in the area postrema.

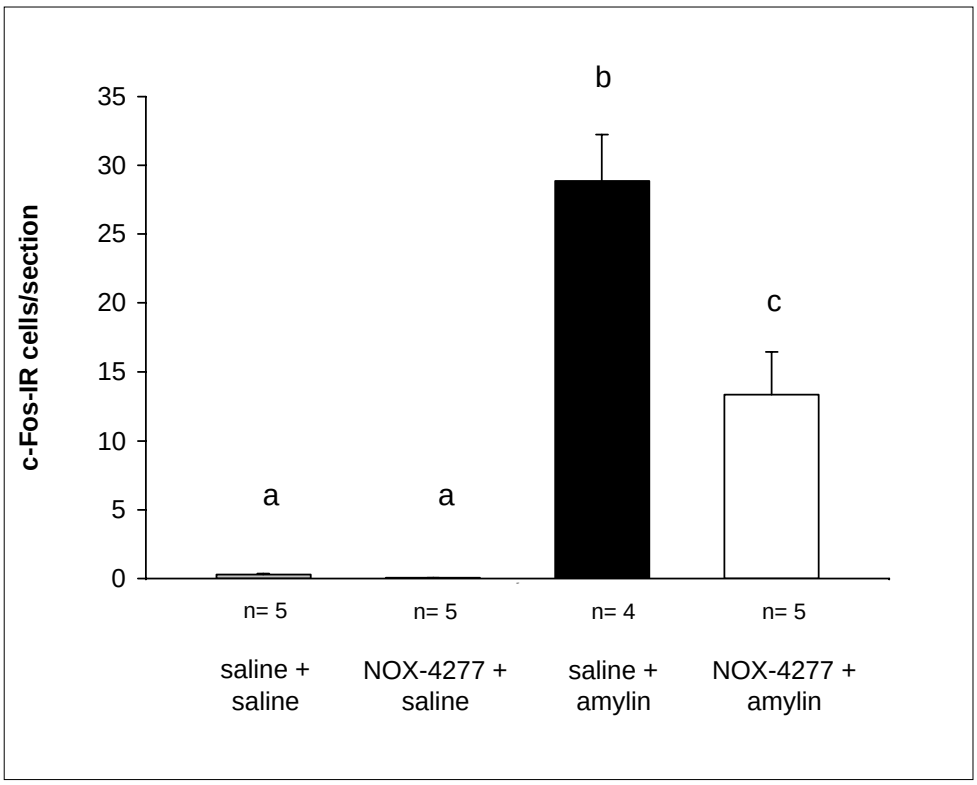

Fig. 3. Quantification of c-Fos-immunoreactive (c-Fos-IR) nuclei in the area postrema of the different experimental groups. Bars represent group means (± SEM). Amylin (20 µg/kg s.c.) elicited a significant increase of c-Fos expression while anti-amylin Spiegelmer NOX-A42 alone had no effect on area postrema neurons. The pre-treatment with NOX-A42 (3 mg/kg i.v.) led to a significant attenuation of the amylin induced c-Fos expression. Bars with different letters are significantly different ($P < 0.05$).

Discussion

In line with previous studies [9, 15] amylin significantly stimulated subfornical organ and area postrema neurons which are considered the main target structures for circulating amylin. As shown by electrophysiological recordings, co-application of the inactive Spiegelmer did not affect amylin`s excitatory responses on subfornical organ neurons. The reason why the latency of the amylin-mediated excitation was not longer under the blockade of amylin, as it may be expected, is unknown. In fact, there rather appeared to be a tendency for a shorter onset, which, however, was not significant. Neither the active nor the inactive control Spiegelmer seemed to produce any toxic or unspecific side effects when superfused alone.

Various amylin receptor antagonists had been useful in identifying amylinergic responses. AC187 is one of the most potent amylin receptor blockers [18]. In the present study we introduced a member of a new class of L-RNA-based hormone antagonists, the anti-amylin Spiegelmer NOX-A42. In contrast to classic hormone antagonists, Spiegelmers do not interact with the hormone receptor but reduce hormone action by a direct binding and thus reduce the biologically active free circulating hormone. As Spiegelmers are L-RNA compounds, they are not targets for nucleases that metabolize D-oligonucleotides [19]. This leads to a tremendous stability of Spiegelmers in biological fluids [19]. Therefore, Spiegelmers are useful antagonists for experimental purposes. The biostable anti-amylin Spiegelmer NOX-A42 could be a promising new tool to chronically block endogenous amylin action without using laborious and more invasive techniques like osmotic minipumps for chronic AC187 infusion. Under certain pathophysiological conditions, excessive amylin release may contribute to disorders in energy homeostasis, e.g. cancer anorexia occuring during certain pancreatic neoplastic diseases that are associated with chronically supraphysiological plasma amylin levels [25]. Amylin inhibition through NOX-A42 could be a promising new approach for the treatment of such disorders.

Conclusion

In summary, the present study provides *in vitro* and *in vivo* evidence that the anti-amylin SPM antagonizes amylinergic responses on neuronal function in the brain. Long acting anti-amylin SPMs may be useful as experimental tools but also as possible therapeutic compounds for disorders characterized by hyperamylinemia.

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